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A COMPARATIVE STUDY OF LEUCOPHORE-ACTIVATING SUBSTANCES FROM THE EYESTALKS OF TWO CRUSTACEANS, *PALAEMONETES VULGARIS* AND *UCA PUGILATOR*[1]

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After the discovery by Koller (1925) that crustacean chromatophores are under endocrine control, a number of investigators found that neuroendocrine tissues from one species of crustacean would activate the chromatophores of another (Fingerman, 1963). For example, Brown and Scudamore (1940) demonstrated that extracts of sinus glands from the prawn, *Palaemonetes vulgaris,* and the fiddler crab, *Uca pugilator,* concentrate the red pigment of the prawn and disperse the melanin of the crab, and that these effects are due to different substances. More recently Fingerman and Couch (1967) showed that although both *Uca* and *Palaemonetes* possess substances that disperse the melanin in *Uca,* disperse the red pigment in both species, and concentrate the red pigment in both species, the substance present in an extract prepared from organs of either species that is effective in either concentrating or dispersing the red pigment of the prawn is different from the substances in the same extract which has the same qualitative effect on the red pigment in the crab.

Much less information, however, is available about leucophores of crustaceans than about the black, brown-black, and red chromatophores. For example, no one has previously determined whether extracts of eyestalks from *Palaemonetes vulgaris* have any effect on the leucophores of *Uca pugilator.* In fact, only recently has evidence been presented for a white pigment-dispersing substance in *Uca pugilator* (Rao, Fingerman and Bartell, 1967). Earlier Sandeen (1950) had reported the presence of a white pigment-concentrating substance in *Uca pugilator.* Brown (1935) found that extracts of eyestalks from *Palaemonetes* cause concentration of its white chromatophoric pigment. However, no evidence is available for the presence of a white pigment-dispersing substance in this genus. In view of the paucity of information about leucophores of crustaceans the present investigation was designed (1) to determine the effect of extracts of eyestalks from *Palaemonetes vulgaris* on the leucophores of *Uca pugilator,* (2) to compare the leucophore-activating substances present in the eyestalks of *Uca* and *Palaemonetes* by means of their behavior during gel filtration, and (3) to obtain further information concerning the antagonism described by Rao, Fingerman and Bartell (1967) between the white pigment-dispersing and -concentrating substances from *Uca.*

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Materials and Methods

The specimens of *Palaemonetes vulgaris* and some of the *Uca pugilator* used in this investigation were collected in the vicinity of Woods Hole, Massachusetts. The remainder of the *Uca pugilator* were from the area of Panacea, Florida. We thank the members of the Supply Department at the Marine Biological Laboratory and the personnel of the Gulf Specimen Company, Panacea, Florida, for their cooperation.

In order to determine the magnitude of the response to a chromatophorotropin the chromatophores were staged until the response had been completed by using the system of Hogben and Slome (1931) in which Stage 1 represents maximal pigment concentration, Stage 5 maximal dispersion, and Stages 2, 3, and 4 the intermediate conditions. The chromatophorotropic activity of the extract could then be expressed in terms of the Standard Integrated Response as defined by Fingerman, Rao and Bartell (1967) which is a measure of both the amplitude and duration of the response.

Extracts were prepared in three different ways from eyestalks of *Uca* as well as *Palaemonetes*. Method I was used for preparing fresh aqueous extracts of the eyestalks. The eyestalks, dissected fresh from the donors, were placed in an embryological dish, triturated with a glass rod, and extracted with the desired volume of either crustacean saline (Pantin, 1934) or distilled water. The extract was centrifuged at $1500 \times g$ and at room temperature for 10 minutes and the supernatant was then used in the experiments. The extracts prepared directly in saline were used for immediate determination of the chromatophorotropic activity of the extracted tissue whereas the extracts prepared directly in distilled water were used in column chromatographic experiments. Method II was used for preparing freeze-dried extracts of the eyestalks. Freshly dissected eyestalks were first extracted in the particular desired volume of distilled water as noted below where the appropriate experiments are described. The extract was then centrifuged at $1500 \times g$ and at room temperature for 10 minutes, and the supernatant was subsequently lyophilized. The freeze-dried material was dissolved in crustacean saline for assay. Method III was used for preparing the ethanol-soluble fraction of the eyestalks. Freshly dissected eyestalks were blotted with a paper towel, then placed in an embryological watch glass, triturated, and extracted in 10 ml of ethanol taking care to avoid excessive stirring during the extraction. The extract was centrifuged at $1500 \times g$ for 10 minutes and the supernatant was decanted into an evaporating dish. The alcohol was then allowed to evaporate at room temperature and the remaining material was eluted in saline for immediate assay or in distilled water or ethanol for use in column chromatographic experiments.

Specimens of *Uca pugilator* were used in the assays for the white pigment-dispersing and -concentrating substances. Eyestalkless crabs, their white pigment is maximally dispersed, were used in the assays for white pigment-concentrating substances, whereas intact fiddler crabs adapted to a black background, their white pigment is maximally concentrated, were used in the assays for white pigment-dispersing substances. In the latter assay only crabs from Panacea were used because they are capable of concentrating their white pigment maximally when adapted to a black background (Rao, Fingerman and Bartell, 1967). In contrast, as Brown and Sandeen (1948) had reported earlier, fiddler crabs from Woods

Hole do not concentrate their white pigment to the maximum extent when adapted to a black background. During the period of assay the crabs were exposed to light intensities of 2.9–3.3 meter-candles and temperatures of 22–24° C. The number of animals used in each assay varied with the experiment and, therefore, will be given at the appropriate places below. Each extract that was assayed was injected in a dose of 0.05 ml per crab.

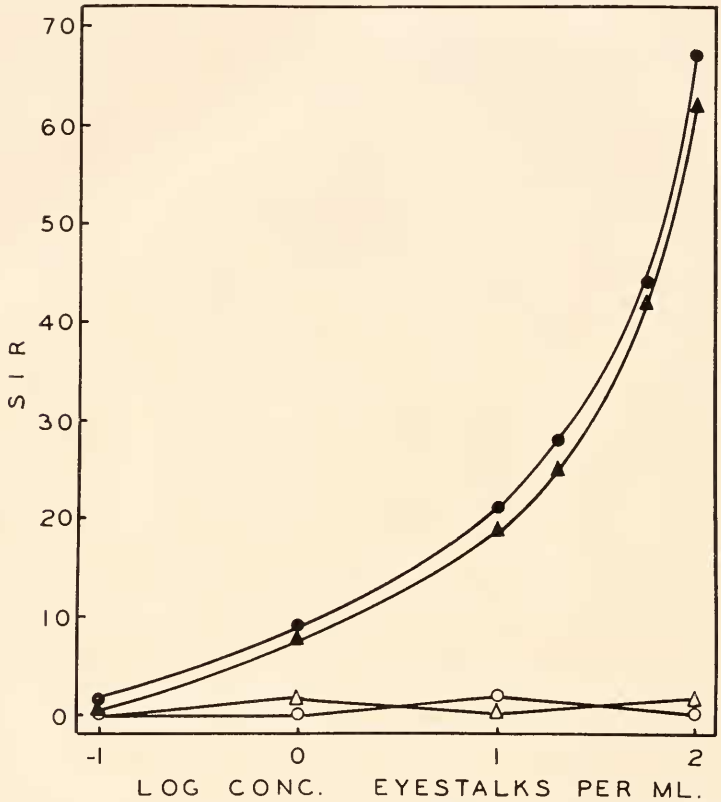

FIGURE 1. Relationships between the Standard Integrated Response (SIR) of the leucophores in *Uca pugilator* and the logarithm of the relative concentration of extracts of the eyestalks from *Uca pugilator*. White pigment-dispersing responses evoked by the extract prepared directly in saline (dots) and the ethanol-soluble fraction (solid triangles); white pigment-concentrating responses evoked by the extract prepared directly in saline (circles) and the ethanol-soluble fraction (empty triangles).

Two types of gels, Bio-Gel P-6 (Calbiochem) and Sephadex LH-20 (Pharmacia Fine Chemicals), were used in the present study for chromatographic separation of leucophore-activating substances. The size of the Bio-Gel column was 27×1.5 cm. The void volume of the column, as determined by filtering Blue Dextran 2000 (Pharmacia Fine Chemicals) through the column, was 14.0 ml. The material to be separated on the Bio-Gel was prepared in a small volume of distilled water (0.2 to 0.4 ml) and applied to the top of the column. Distilled

water was used as the solvent. Two ml fractions were collected. Before being assayed each fraction was made isosmotic to the blood of the fiddler crab by adding one part of 400% crustacean saline to three parts of the eluted fraction. Each fraction was assayed on three eyestalkless crabs and three crabs adapted to a black background.

Sephadex LH-20 was equilibrated with ethanol which was used as the solvent. The size of the column was 28 × 1.5 cm, the void volume of the column was 21 ml. The ethanol extract (sample size 0.2 to 0.4 ml) was applied to the top of the column and then the solvent was allowed to flow through. Two ml fractions were collected. The alcohol in each sample was allowed to evaporate at room temperature after which the material in each sample was dissolved in 0.5 ml of crustacean saline and assayed on three eyestalkless *Uca* and three intact *Uca* adapted to a black background.

Experiments and Results

The aim of the first series of experiments was to determine and compare the relationships between the standard integrated pigment-dispersing and -concentrating responses of leucophores in *Uca pugilator* and the concentration of eyestalk extracts prepared according to Methods I and III from the prawn and the crab. The fresh saline extract of crab eyestalks was prepared from 100 eyestalks dissected one each from 100 *Uca* and extracted in 1 ml of saline. The ethanol-soluble fraction was prepared from the contralateral eyestalks from the same 100 crabs. The ethanol-soluble fraction was likewise eluted in 1 ml of saline. A series of six dilutions from 100 to 0.1 eyestalks per ml was prepared from each of these extracts by using saline as the diluent. In similar fashion a saline extract and the ethanol-soluble fraction were prepared with eyestalks of *Palaemonetes*. In each experiment the extract of a given concentration was assayed on five eyestalkless *Uca* and five intact *Uca* adapted to a black background. Each experiment was repeated once. The averaged results shown in Figures 1 and 2 reveal that for both species the extract prepared directly in saline and the ethanol-soluble fraction of the eyestalks evoked nearly identical white pigment-dispersing activities at all concentrations tested. The aqueous extracts of eyestalks from *Uca* and *Palaemonetes*, and the ethanol-soluble fraction of the eyestalks from *Uca* failed to concentrate the initially dispersed white pigment in the chromatophores of *Uca*. However, the ethanol-soluble fraction of the eyestalks from *Palaemonetes* evoked white pigment concentration in *Uca*. The latter response was highest at the lowest concentration of the extract tested and decreased with increase in the concentration of the extract (Fig. 2).

The second series of the experiments was conducted to determine whether the technique of gel filtration would be helpful in determining whether the white pigment-dispersing substances from the prawn and the crab are identical or not. Fifty eyestalks, freshly dissected from *Uca*, were extracted in 0.4 ml of distilled water. After centrifugation the supernatant was fractionated on a column of Bio-Gel P-6. The white pigment-dispersing activity appeared at two places, fractions 7 and 12 (Fig. 3). The activity of fraction 12 is eight to ninefold higher than that of fraction 7. No white pigment concentration was detected. Similar results were obtained when distilled water extracts of 50 eyestalks from *Palae-*

monetes, and a mixture of the distilled water extracts from 25 eyestalks of *Uca* and 25 eyestalks of *Palaemonetes* were chromatographed on the column of Bio-Gel P-6 (Fig. 3). The above experiments were repeated twice with consistent results. On the basis of these experiments there is no reason to consider the white pigment-dispersing substances from the prawn and the crab are anything but identical.

In the next experiment the ethanol-soluble fraction of 50 freshly dissected eyestalks from *Uca* was prepared taking care to avoid excessive stirring during the extraction. After the alcohol had evaporated the material was eluted in 0.4 ml distilled water and subjected to chromatography on the Bio-Gel column. The

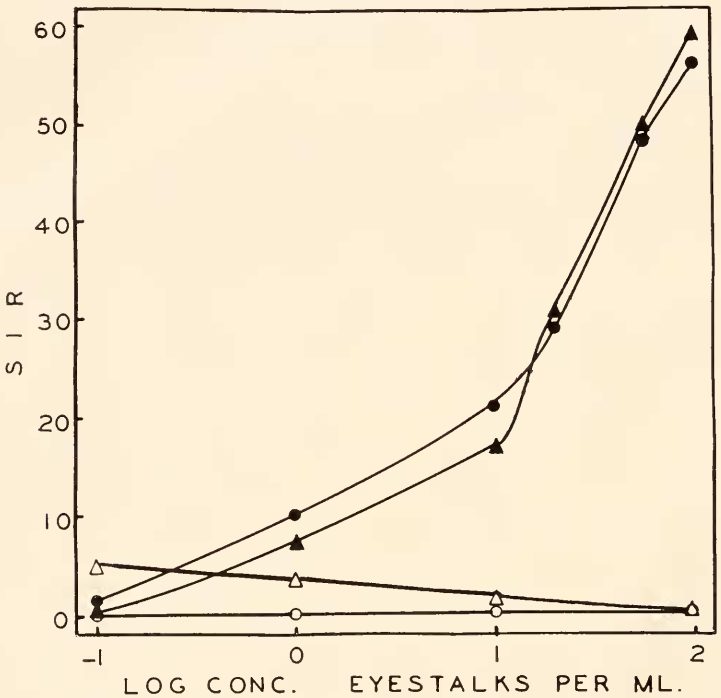

FIGURE 2. Relationships between the Standard Integrated Response (SIR) of the leucophores in *Uca pugilator* and the logarithm of the relative concentration of extracts of the eyestalks from *Palaemonetes vulgaris.* See Figure 1 for key to symbols.

white pigment-dispersing activity appeared at one place, fraction 7 (Fig. 4). This fraction also had most of the red pigment which is always extracted from the eyestalks along with the chromatophorotropins. The substances in fraction 7 were excluded from this gel which has an exclusion limit of 4600 daltons. No white pigment concentration was observed in this experiment also. Similar results were obtained when a distilled water eluate of the ethanol-soluble fraction from 50 eyestalks of *Palaemonetes* was chromatographed (Fig. 4).

Previous investigation (Bartell, Rao and Fingerman, 1967) revealed that when aqueous and alcohol extracts of eyestalks from *Uca* were filtered through Bio-Gel P-6 the melanin-dispersing activity appeared at two peaks, one in the void volume

and the other at an R_f of 0.6. The first peak appeared to be due to the melanin-dispersing substance complexed with a lipid-containing material of high molecular weight and the R_f 0.6 peak of free peptide. By merely stirring the extract it was possible to split the complex and free the smaller component. To determine whether a similar complex accounts for the white pigment-dispersing activity recovered in the void volume of the Bio-Gel column the following experiment was performed.

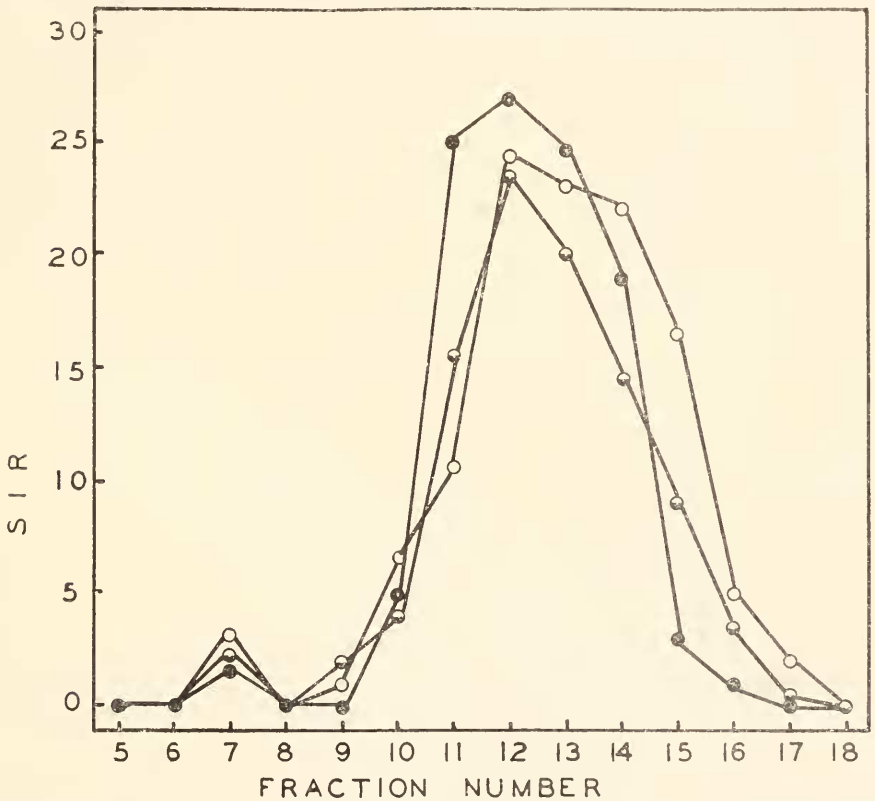

FIGURE 3. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the fractions of extracts of eyestalks prepared directly in distilled water from *Uca* (dots) and *Palaemonetes* (circles), and of the mixture of extracts of the eyestalks from *Uca* and *Palaemonetes* (half-filled circles) after filtration through a 27×1.5 cm column of Bio-Gel P-6. Flow rate: 48 ml per hour. Before assay the samples were made isosmotic to the blood of the fiddler crab. See text for details.

Fifty eyestalks of *Uca* were extracted in ethanol and during the process of extraction the extract was vigorously stirred with a glass rod. After the alcohol had evaporated from the ethanol-soluble fraction the material was eluted in 0.4 ml distilled water and chromatographed on Bio-Gel P-6. Using the same procedure an extract was made from 50 eyestalks of *Palaemonetes* and likewise chromatographed. The results shown in Fig 4 reveal that the white pigment-dispersing activity appeared at two peaks, fractions 7 and 12. These results indicate that

the white pigment-dispersing substance in the ethanol fraction prepared without vigorous stirring is indeed also loosely bound to a heavier substance. The next set of experiments was designed to determine the chromatographic behavior of the white pigment-dispersing substance in the ethanol-soluble fraction prepared from eyestalks pretreated with 10 ml acetone or chloroform. In the first experiment of this series 50 eyestalks of *Uca* were extracted in acetone. The acetone-soluble fraction which contains a white pigment-concentrating substance, but none of the

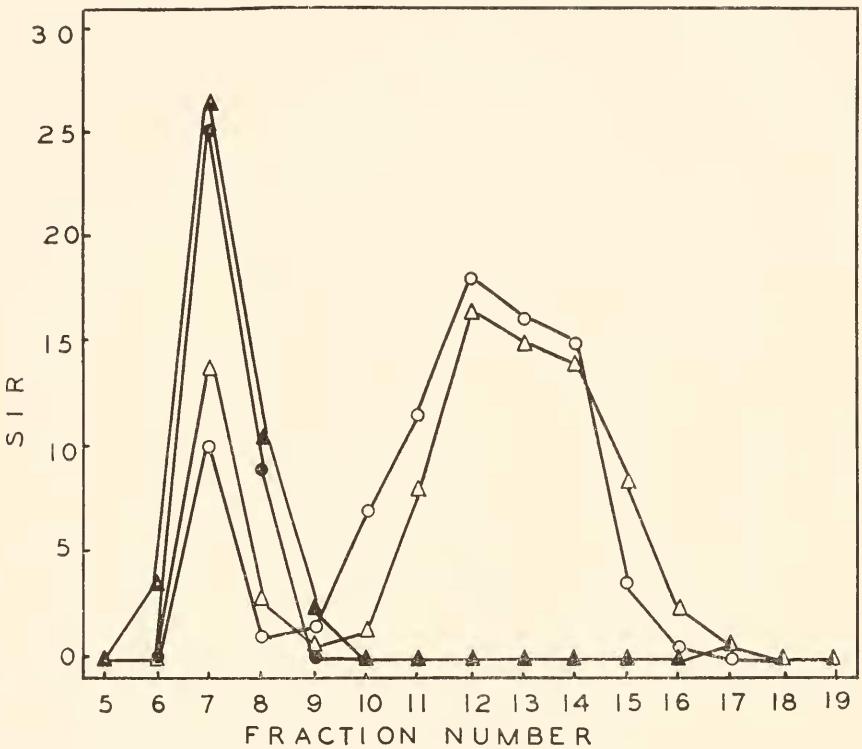

FIGURE 4. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the fractions of the distilled water eluates of the ethanol-soluble fraction extracted without excessive stirring from the eyestalks of *Uca* (dots) and *Palaemonetes* (solid triangles) and of the ethanol-soluble fractions extracted with excessive stirring from the eyestalks of *Uca* (circles) and *Palaemonetes* (empty triangles) after filtration through a column of Bio-Gel P-6. Other details same as Figure 3.

white pigment-dispersing material (Rao, Fingerman and Bartell, 1967) was discarded. The acetone-insoluble material was dried in a desiccator and then extracted in 10 ml ethanol. The alcohol in the ethanol-soluble fraction was allowed to evaporate at room temperature, then the material was eluted in 0.4 ml of distilled water and chromatographed on Bio-Gel. Similarly an ethanol-soluble fraction of the chloroform-insoluble fraction of 50 eyestalks from *Uca* was prepared and chromatographed. The results shown in Figure 5 for the chloroform-treated and acetone-treated material reveal the absence of activity in the samples from the void

volume, but the activity appeared later and peaked in fraction 12. Thus, by treatment with chloroform or acetone it was possible to remove the white pigment-dispersing hormone from the heavy component in the ethanol-soluble material.

The aim of the next experiment was to determine the effect of boiling on the white pigment-dispersing substances in an extract prepared directly in water and in the ethanol-soluble fraction of the eyestalks of *Uca* and of *Palaemonetes* as measured by changes in the responses to these substances. Distilled water ex-

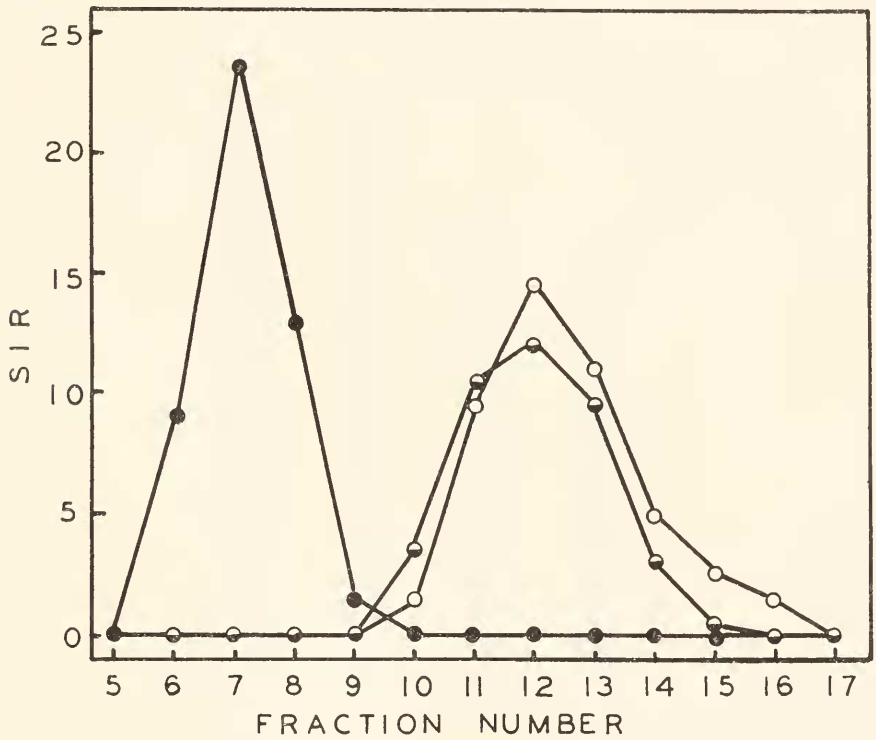

FIGURE 5. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the various fractions of the distilled water eluates of the ethanol-soluble fraction prepared from fresh eyestalks of *Uca* (dots) and of the distilled water eluates of the ethanol-soluble fraction prepared from the eyestalks of *Uca* that were pre-extracted with acetone (circles) or chloroform (half-filled circles) after filtration through a column of Bio-Gel P-6. Other details same as Figure 3.

tracts were made from 50 eyestalks of *Palaemonetes* and 50 eyestalks of *Uca*. The extracts were filtered individually through the column of Bio-Gel P-6 and fraction 12, which produced the most white pigment-dispersing activity of the material retarded by the gel (Figs. 3, 4, 5), was divided into two equal portions. One portion of the extract was placed in a boiling water bath for five minutes while the other portion was left at room temperature. The boiled extract was cooled, made up to the original volume, and then the boiled and unboiled extracts were each assayed for white pigment-dispersing activity on 10 intact crabs adapted to

a black background. Ethanol-soluble fractions of 50 eyestalks of *Uca* and 50 eyestalks of *Palaemonetes* were then prepared. During the extraction vigorous stirring was employed so as to liberate the white pigment-dispersing substance from the heavy component. After filtering these extracts through Bio-Gel P-6 fraction 12 was boiled as in the previous experiment and assayed for white pigment-dispersing activity. Each experiment was repeated once and the averaged results are shown in Figure 6. The white pigment-dispersing substance in the ethanol-soluble fraction of the eyestalks from *Uca* as well as *Palaemonetes* is thermolabile, whereas the white pigment-dispersing substance in an extract of these eye-

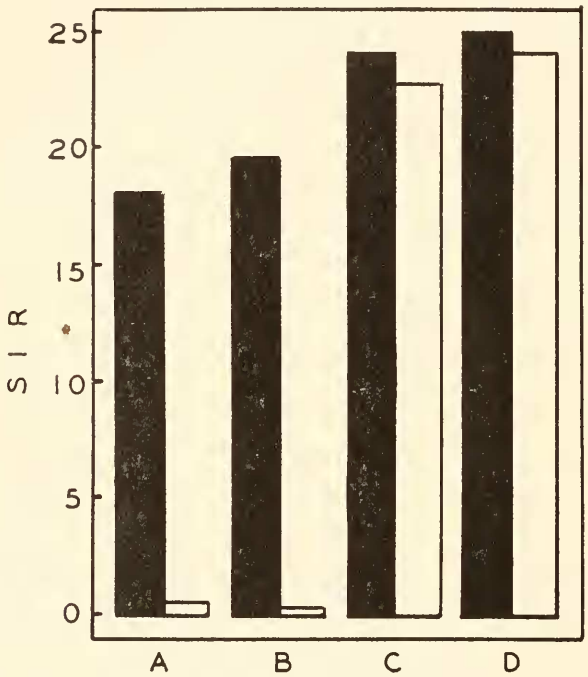

FIGURE 6. The white pigment-dispersing Standard Integrated Repsonses (SIR) evoked by the unboiled (solid bars) and boiled (empty bars) fractions retarded by Bio-Gel P-6. Distilled water eluates of the ethanol-soluble fraction of the eyestalks from *Palaemonetes* (A) and *Uca* (B); distilled water extracts of the eyestalks from *Palaemonetes* (C) and *Uca* (D).

stalks prepared directly in water is thermostable. The most logical explanation of these results is that we are dealing with at least two different white pigment-dispersing subsances; one, thermostable, is extractable in distilled water, the other, thermolabile, is extractable in ethanol.

Previous investigation (Rao, Fingerman and Bartell, 1967) revealed that the white pigment-dispersing substance antagonizes the action of the white pigment-concentrating substance. If a white pigment-concentrating substance is present in the ethanol-soluble fraction of eyestalks from *Uca* in spite of the fact that its presence is not revealed following injection of such extracts into eyestalkless crabs (Fig. 1), and if it is thermostable, its presence should become apparent by selec-

tively inactivating the antagonistic thermolabile white pigment-dispersing substance by boiling. The following experiment was conducted to test this conclusion. One hundred twenty eyestalks from *Uca* were extracted in 24 ml ethanol and after centrifugation 22 ml of the supernatant were divided equally among 11 evaporating dishes. The alcohol was allowed to evaporate at room temperature. Then one dish was placed in a desiccator while the others were placed in an oven at 95° C. The dishes were removed one at a time, 2, 4, 6, 8, 12, 14, 16, 18, 24, and 36 hours

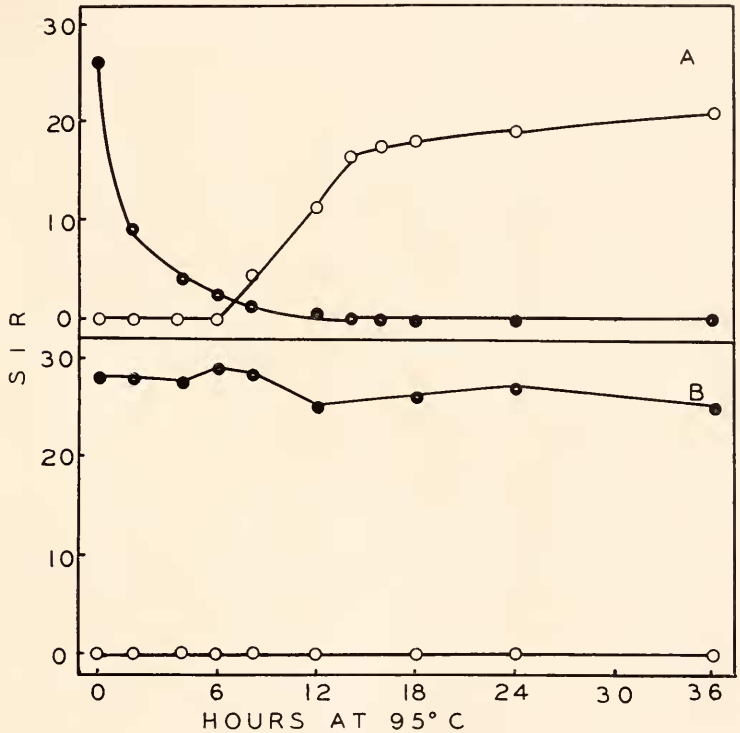

FIGURE 7. The effect of drying the ethanol-soluble fraction of fresh eyestalks of *Uca* (A) and the lyophilized water-soluble fraction of the eyestalks of *Uca* (B) for varying lengths of time in an oven. Dots, white pigment-dispersing Standard Integrated Responses (SIR); circles, white pigment-concentrating Standard Integrated Responses. The concentration of each extract was one eyestalk per dose of 0.05 ml.

after they had been placed in the oven. After removal from the oven the sample was placed in a desiccator so that all the samples could be assayed on the same day. Each sample was dissolved in 0.5 ml saline and tested on five eyestalkless *Uca* and five intact *Uca* adapted to a black background. The experiment was repeated once. An aqueous extract was then prepared directly from 120 eyestalks of *Uca* also. This extract was likewise divided into 11 portions of 2 ml each, but they were lyophilized before being treated as in the protocol of the above experiment. The averaged results for these experiments are shown in Figure 7. With increase in the length of exposure to heat the white pigment-dispersing activity

decreased and completely disappeared in the samples heated for 12 hours and longer. However, concomitantly the white pigment-concentrating activity began to appear and gradually increased with decrease of the white pigment-dispersing activity. In contrast, the material in the extract prepared directly in water always evoked only dispersion of the white pigment. Even if the white pigment-concentrating substance is present in the material extracted directly in water, it is not possible to demonstrate its activity by this method because the antagonistic white pigment-dispersing substance in this fraction is thermostable.

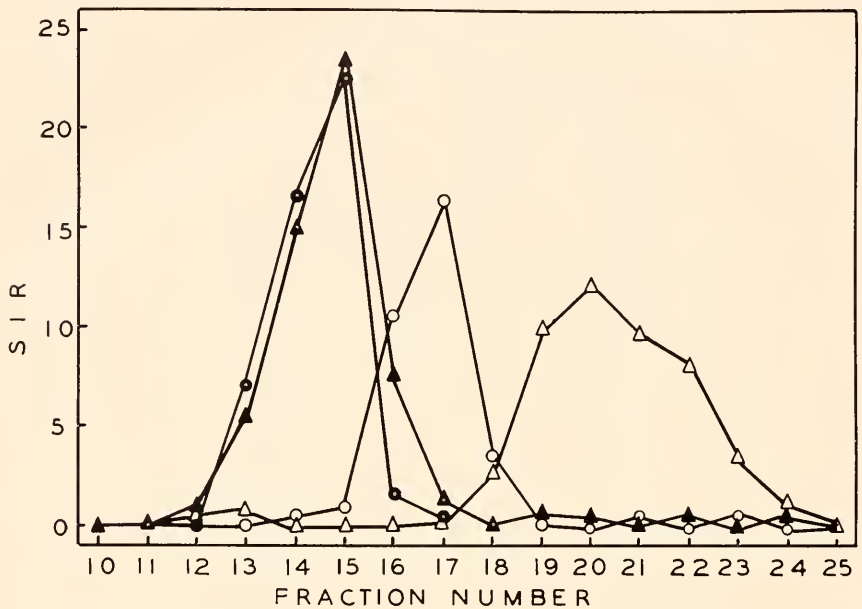

FIGURE 8. The white pigment-dispersing (dots and solid triangles) and white pigment-concentrating (circles and empty triangles) Standard Integrated Responses (SIR) evoked by the fractions of the ethanol-soluble material extracted from the eyestalks of *Uca* (dots and empty triangles) separated by filtration through a 28 × 1.5 cm column of Sephadex LH-20. Flow rate: 30 ml per hour. Sample size: 2 ml.

From the above experiment it is clear that the ethanol-soluble fraction of the eyestalks from *Uca* has both the white pigment-dispersing and -concentrating substances. The ethanol-soluble fraction of the eyestalks from *Palaemonetes* also has both substances (Fig. 2). However, the white pigment-concentrating substance did not appear in any of the fractions obtained after chromatographing the ethanol-soluble fractions of eyestalks from *Uca* and *Palaemonetes* on the Bio-Gel. Consequently in an effort to separate the white pigment-dispersing and -concentrating substances and obtain an estimate of their relative molecular weight it was decided to try another gel. Ethanol extracts were consequently chromatographed on Sephadex LH-20 with ethanol as the solvent. Fifty eyestalks from *Uca* were extracted in 10 ml ethanol and the ethanol-soluble fraction was allowed to evaporate at room temperature. The material was redissolved in 0.4 ml ethanol and then

chromatographed on a column of LH-20 in ethanol. The red pigment in the extract peaked in fraction 11 which represents the end of the void volume, and this sample had no effect on the white chromatophores of *Uca* (Fig. 8). The white pigment-dispersing activity peaked at fraction 15, whereas the white pigment-con-

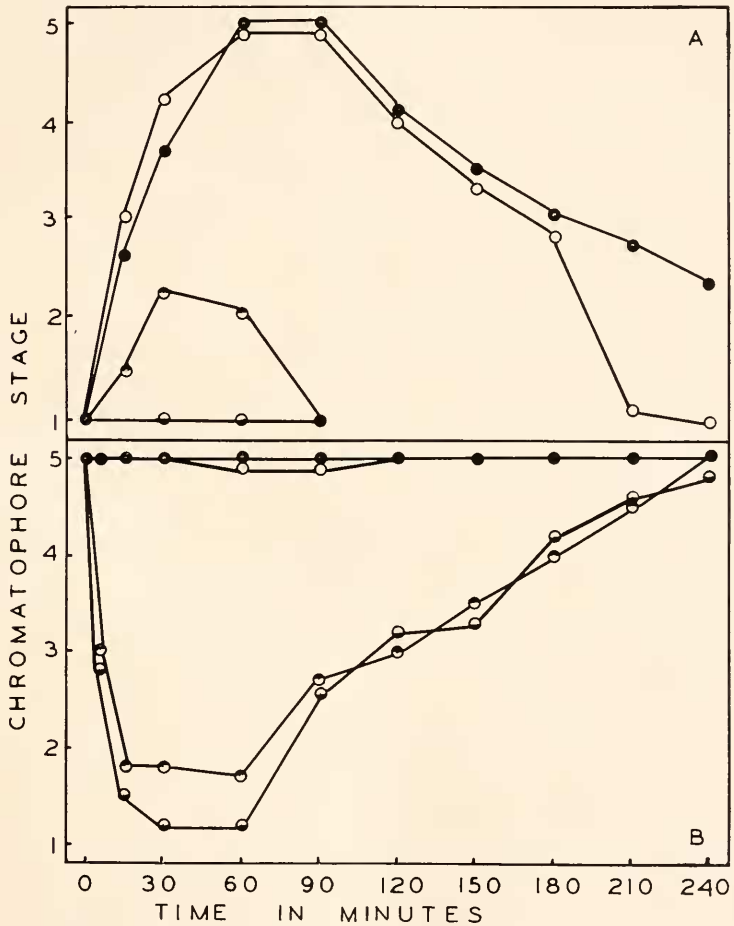

FIGURE 9. The white pigment-dispersing (A) and white pigment-concentrating (B) responses evoked by saline eluates of the untreated ethanol-soluble fraction (dots), isopropyl ether-treated ethanol-soluble fraction (circles with bottom half filled), untreated lyophilized fraction extracted directly in distilled water (circles), and the isopropyl ether-treated lyophilized fraction extracted directly in distilled water (circles with top half filled) from fresh eyestalks of *Uca pugilator*. The concentration of each extract tested was 1 eyestalk per dose of 0.05 ml.

centrating activity which was then apparent peaked at fraction 17; the substances had been separated. In the next experiment the ethanol-soluble fracion prepared from 50 eyestalks of *Palaemonetes* was filtered through LH-20. The peak of white pigment-dispersing activity coincided with that of the ethanol-soluble fraction of eyestalks from *Uca*. However, the white pigment-concentrating activity in the

extracts of eyestalks from *Palaemonetes* peaked in fraction 21, much later than did the white pigment-concentrating substance from *Uca* (Fig. 8).

So far no evidence has been obtained as to whether or not a white pigment-concentrating substance is present in extracts of eyestalks from *Uca* prepared in water. Rao, Bartell and Fingerman (1968) showed that the melanin-dispersing substances in the extract prepared directly in water and in the ethanol-soluble fraction of eyestalks from *Uca* were inactivated by isopropyl ether treatment. The following experiment was conducted to determine (a) whether the white pigment-dispersing substances in these extracts can also be inactivated in a similar way and (b) whether the extract prepared directly in water contains both the white pigment-dispersing and -concentrating substances. An extract was prepared directly in 15 ml distilled water from 40 eyestalks of *Uca* and divided into two equal portions which were subsequently lyophilized. One sample which served as the control was stored on dry ice. To the other sample was added 10 ml isopropyl ether and the container was then covered. After an overnight exposure to isopropyl ether the ether was allowed to evaporate at room temperature and the material was dissolved in 1.0 ml saline and injected into 10 eyestalkless crabs and 10 intact crabs adapted to a black background. The control sample was likewise assayed. The ethanol-soluble fraction was then prepared from 40 eyestalks and divided into two equal portions. After the alcohol had evaporated one sample was placed in a desiccator, while the other was treated with isopropyl ether as described above. These treated and untreated samples were then assayed for action on the white chromatophores. The results (Fig. 9) reveal that the white pigment-dispersing substances in the ethanol-soluble fraction of the eyestalks from *Uca* and in the extract prepared directly in water are inactivated by isopropyl ether treatment just as are the melanin-dispersing substances. In the absence of the antagonistic white pigment-dispersing substance, the white pigment-concentrating substance was demonstrable. Therefore, it is clear that the white pigment-dispersing and -concentrating substances are both present in the aqueous and alcoholic extracts of these eyestalks.

Discussion

A previous comparative study (Fingerman and Couch, 1967) of chromatophorotropins from *Uca* and *Palaemonetes* revealed that extracts of eyestalks from *Palaemonetes* evoke pigment migration in the melanophores and erythrophores of *Uca*. The present study revealed that extracts of eyestalks from *Palaemonetes* can evoke dispersion and concentration of the pigment in the leucophores of *Uca* also. Of significance is the question whether pigment dispersion in each type of chromatophore is regulated by a separate substance or whether a single substance evokes pigment dispersion in the melanophores, erythrophores, and leucophores. Brown and Fingerman (1951) have shown that the melanin-dispersing and red pigment-dispersing substances in the supraesophageal ganglia of *Uca* are different. Fingerman and Couch (1967) arrived at the same conclusion for the red pigment-dispersing and melanin-dispersing substances in the eyestalk of *Uca*. Rao, Fingerman and Bartell (1967) found that an extract of the circumesophageal connectives from *Uca* evoked melanin dispersion but not white pigment dispersion in *Uca*, and concluded that it is highly unlikely that both actions are due to a single

substance. The latter investigators also found that the white pigment-concentrating substance antagonizes the action of white pigment-dispersing substance but not the melanin-dispersing substance. Further support for the conclusion that dispersion of white pigment and dispersion of melanin are due to different substances comes from the present study. At concentrations of 0.5 eyestalk per dose and above the melanin-dispersing activity of the ethanol-soluble fraction of the eyestalks from *Uca* is much higher than that of an aqueous extract prepared directly from the eyestalks of *Uca* (Rao, Bartell and Fingerman, 1967). In contrast, at all the concentrations tested the white pigment-dispersing activity of the ethanol-soluble fraction of the eyestalks was nearly identical to that of the aqueous extract of the eyestalks (Figs. 1, 2). This observation is also quite different from the situation reported for the crab, *Ocypode macrocera,* where with a concentration of one eyestalk per dose the white pigment-dispersing activity of the ethanol-soluble fraction was 25 times more than that evoked by an extract of the optic ganglia prepared direcly in saline (Rao, 1967). *Uca* and *Ocypode* are members of the same family, the Ocypodidae. Attempts are now underway in this laboratory to separate the melanin-dispersing and white pigment-dispersing substances of *Uca pugilator.*

Injection of an extract of eyestalks from *Uca* prepared directly in saline results in dispersion but not concentration of the pigment in the leucophores of *Uca.* However, acetone fractionation revealed that the white pigment-dispersing and -concentrating substances are both present in the eyestalk (Rao, Fingerman and Bartell, 1967). Two alternative explanations were given. (1) A white pigment-concentrating substance is present in the aqueous extract but its expression is inhibited by the presence of its antagonist, the white pigment-dispersing substance or (2) a white pigment-concentrating substance exists in the nervous tissues in an inactive form and acetone extraction renders it active and soluble in water. The present study lends support to the former view. At all concentrations of the extracts prepared directly in water and the ethanol-soluble fractions of the eyestalks from *Uca* and the extracts of eyestalks from *Palaemonetes* prepared directly in water the white pigment-dispersing substance dominated the white pigment-concentrating substance (Figs. 1, 2). However, at low concentrations of the ethanol-soluble material from the eyestalks of *Palaemonetes* white pigment-concentration was evident, and as the concentration increased the white pigment-dispersing substance dominated more and more the white pigment-concentrating substance (Fig. 2).

The white pigment-concentrating substance in the eyestalk extracts could not be recovered after chromatography on Bio-Gel P-6. This substance was probably adsorbed to the gel matrix. However, chromatography of ethanol extracts of the eyestalks on Sephadex LH-20 yielded good separation of the white pigment-dispersing and -concentrating substances (Fig. 8). The white pigment-concentrating substance in the eyestalks of *Palaemonetes* is a smaller molecule than the white pigment-concentrating substance in the eyestalks of *Uca.* The gel filtration studies indicate that the low molecular weight substances having white pigment-dispersing activity from *Uca* and *Palaemonetes* may not differ in their molecular weights. The observation (Fig. 6) that the white pigment-dispersing substance in the ethanol-soluble fraction is thermolabile while that in the material extracted directly in water is thermostable leads to the conclusion that we are dealing with at least two different substances.

The finding that when distilled water eluates of the ethanol-soluble fraction are filtered through Bio-Gel P-6 (Fig. 4) most of the white pigment-dispersing activity is associated with the material in the void volume is in agreement with previous work on melanin-dispersing substances (Bartell, Rao and Fingerman, 1967). These investigators obtained evidence in favor of the theory that the high molecular weight material in the void volume consists of a complex of an active peptide and a large lipoidal substance. Rao, Bartell and Fingerman (1968) have suggested that this lipid-containing material may be the carrier substance whose presence and nature has been revealed by cytochemical studies of neurosecretory cells in other organisms. Bern and Hagadorn (1965) have reviewed the available information on the chemical nature of the carrier substance. When ethanol extracts were chromatographed on LH-20 (Fig. 8) no activity was found in the void volume, showing that the material while in ethanol is a small molecule.

Although certain substances in the eyestalks of *Palaemonetes* evoke pigment migration in the leucophores of *Uca*, we are unable to determine whether they act on the leucophores of *Palaemonetes* itself. The responses seen in this laboratory of the leucophores in *Palaemonetes* to eyestalk extracts have proven to be extremely erratic and as a consequence no definitive conclusions could be drawn from the data. However, after we are able to develop a suitable technique for investigating the responses of the white chromatophores in *Palaemonetes* further comparative study of the control of the leucophores in these two crustaceans would be worthwhile.

Summary

1. The relationships between the response of the leucophores in *Uca pugilator* and the concentration of extracts of eyestalks from *Uca pugilator* and *Palaemonetes vulgaris* were determined. For the first time evidence is provided to show that substances capable of evoking pigment dispersion and pigment concentration in the leucophores of *Uca* are present in the eyestalks of *Palaemonetes*. Contrary to the situation recorded previously for melanin-dispersing activity, extracts prepared directly in saline and the ethanol-soluble fractions of the eyestalks from *Uca* evoked nearly identical white pigment-dispersing activity at all dilutions tested.

2. White pigment-dispersing and -concentrating substances can both be demonstrated to be present in the ethanol-soluble fraction of the eyestalks of *Uca* as well as in the material directly extractable in water in spite of the fact that when these extracts are assayed immediately after preparation they evoke only white pigment dispersion because the white pigment-dispersing substance antagonizes the action of the white pigment-concentrating substance.

3. The eyestalks of *Uca* as well as *Palaemonetes* contain at least two white pigment-dispersing substances each. The substance in the ethanol-soluble fraction is thermolabile whereas that in the extract prepared by extracting the eyestalks directly in saline is thermostable. However, both are inactivated by prolonged exposure to isopropyl ether.

4. Gel-filtration studies revealed that the white pigment-concentrating substance in the ethanol-soluble fraction of the eyestalks of *Palaemonetes* is a smaller molecule than the white pigment-concentrating substance in the ethanol-soluble fraction of the eyestalks of *Uca*, but there is no reason to consider the correspond-

ing white pigment-dispersing substances in the extracts prepared directly in saline, and in the ethanol-soluble fractions from both species as different from each other.

LITERATURE CITED

Bartell, C. K., K. R. Rao and M. Fingerman, 1967. An analysis of the melanin-dispersing activity of aqueous and alcoholic extracts of eyestalks from the fiddler crab, *Uca pugilator*, by means of gel filtration and ultracentrifugation. *Biol. Bull.*, **133**: 458.

Bern, H. A., and I. R. Hagadorn, 1965. Neurosecretion, pp. 354–429. *In:* T. H. Bullock and G. A. Horridge, Eds. *Structure and Function in the Nervous Systems of Invertebrates, Vol. I.* W. H. Freeman and Co., San Francisco.

Brown, F. A., Jr., 1935. Control of pigment migration within the chromatophores of *Palaemonetes vulgaris*. *J. Exp. Zool.* **71**: 1–15.

Brown, F. A., Jr., and M. Fingerman, 1951. Differentiation of black- and red-dispersing factors from the brain of the fiddler crab, *Uca*. *Fed. Proc.*, **10**: 20–21.

Brown, F. A., Jr., and M. I. Sandeen, 1948. Responses of the chromatophores of the fiddler crab, *Uca*, to light and temperature. *Physiol. Zoöl.*, **21**: 361–370.

Brown, F. A., Jr., and H. Scudamore, 1940. Differentiation of two principles from the crustacean sinus gland. *J. Cell. Comp. Physiol.* **15**: 103–119.

Fingerman, M., 1963. *The Control of Chromatophores.* Pergamon-Macmillan, New York, 184 pp.

Fingerman, M., and E. F. Couch, 1967. Differentiation of chromatophorotropins from the prawn, *Palaemonetes vulgaris*, and the fiddler crab, *Uca pugilator*. *J. Exp. Zool.*, **165**: 183–194.

Fingerman, M., K. R. Rao and C. K. Bartell, 1967. A proposed uniform method of reporting response values for crustacean chromatophorotropins: the Standard Integrated Response. *Experientia*, **23**: 962.

Hogben, L., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B.*, **108**: 10–53.

Koller, G., 1925. Farbwechsel bei *Crangon vulgaris*. *Verh. Dtsch. Zool. Ges.*, **30**: 128–132.

Pantin, C. F. A., 1934. The excitation of crustacean muscle. *J. Exp. Biol.*, **11**: 11–27.

Rao, K. R., 1967. Studies on the differentiation of the chromatophorotropins of the crab, *Ocypode macrocera* H. Milne Edwards. *Physiol. Zoöl.*, **40**: 361–370.

Rao, K. R., C. K. Bartell and M. Fingerman, 1967. Relationship between the response of melanophores in the fiddler crab, *Uca pugilator*, and the concentration of eyestalk extract. *Z. Vergl. Physiol.*, **56**: 232–236.

Rao, K. R., C. K. Bartell and M. Fingerman, 1968. Solubility and stability properties of the melanin-dispersing substances from the eyestalks of the fiddler crab, *Uca pugilator*. *Z. Vergl. Physiol.*, **60**: 1–13.

Rao, K. R., M. Fingerman and C. K. Bartell, 1967. Physiology of the white chromatophores in the fiddler crab, *Uca pugilator*. *Biol. Bull.*, **133**: 606–617.

Sandeen, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator*, with special reference to their origins and actions. *Physiol. Zoöl.*, **23**: 337–352.